Unusually fine-scale genetic structuring found in rapidly speciating Malawi cichlid fishes

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SUMMARY

Mechanisms behind the explosive radiation of over 500 cichlid fish species from a single founding population in Lake Malawi during the last 700 000 years are poorly understood. Recent studies have suggested that the degree of population subdivision among the habitat patches within the lake may be responsible, but the evidence has been circumstantial: lack of a dispersal stage in haplochromine cichlids; genetic and colour variation among populations separated by large-scale geographical barriers; and fluctuating lake levels. One reason for the rapidity of speciation in these fishes may be that population subdivision is on a much finer scale than previously thought. Here we quantify the level of population subdivision and estimate migration at a scale of 700–1400 m, in order to investigate whether cichlid populations are sufficiently isolated from each other for allopatric divergence and perhaps speciation to take place. Using six microsatellite loci, we demonstrate the existence of highly significant genetic differentiation between subpopulations on adjacent headlands in each of four rock-dwelling haplochromine cichlid species. Our results suggest that these fish populations are divided into thousands of subunits among which genetic divergence is currently occurring, and that this may provide unprecedented opportunities for allopatric speciation.

1. INTRODUCTION

Rich species flocks have evolved in a number of the world's large water basins, including the three African great lakes (Malawi, Tanganyika and Victoria), Lake Baikal, and the Caspian Sea (Fryer 1996), the most striking example being the haplochromine cichlid fishes of the African lakes. Lake Malawi alone harbours over 500 endemic cichlid species, all maternal mouth-brooders lacking a planktonic larval stage (Ribbink *et al.* 1983). Among these are a largely monophyletic (Meyer 1993) guild of at least 200 small, rock-dwelling species (mbuna), classified into ten genera (Ribbink *et al.* 1983). Though most of these are not formally recognized as species, we have used DNA microsatellites and field observations to demonstrate that interbreeding between sympatric colour forms is absent or only minimal (van Oppen *et al.* 1998). The lack of a dispersal stage in the mbuna, together with their inability to penetrate to depths beyond 25–50 m (Ribbink *et al.* 1983) and their geographical colour variation, has led researchers to suggest that the alternation of rocky headlands with sandy bays along the shore of Lake Malawi may provide numerous geographic barriers to these fishes (Fryer & Iles 1972; Ribbink *et al.* 1983;

McKaye & Gray 1984; Snoeks *et al.* 1994), increasing the possibilities for allopatric speciation. However, no direct evidence exists to support this hypothesis. Previous studies of mbuna have demonstrated genetic differences between allopatric populations, but these populations were separated by large distances of hundreds of kilometres (McKaye *et al.* 1984; Moran & Kornfield 1995), or located on islands that are surrounded by deep troughs (Bowers *et al.* 1994; Moran & Kornfield 1995). It is not known how far individuals disperse, whether adjacent habitat patches are isolated from each other, or whether populations in the lake are subdivided into tens, hundreds or thousands of independent units.

Microsatellites or simple sequence repeats (SSRs) are 16 bp long stretches of sequence that are tandemly repeated throughout the nuclear genome of most eukaryotes (Jarne & Lagoda 1996). They are also found in chloroplast genomes. Mutation rates are in the order of 10^{-2} – 10^{-5} in mammals (Dallas 1992; Weber & Wong 1993; Ellegren 1995; Jin *et al.* 1996), and most microsatellite loci are highly polymorphic, exhibiting many alleles and high heterozygosities. It is believed that the majority of mutations occur by replication slippage (Schlötterer & Tautz 1992), which leads to the addition or deletion of a single repeat unit, but larger deletions and insertions are known to occur as

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Figure 1. (a) Map of Lake Malawi showing the collection area Nkhata Bay. The inset is an enlargement of the Nkhata Bay area showing the four collection sites: Chin, Chindazowa Point; Chir, Chirundu Point; Nku, Nkukuti Point; Mwa, Mwafufu Rock. Stretches of sand/marsh shoreline are marked with grey bars. (b) Allele frequency distribution at locus Pzeb2 for the four *P. (M.) callainos* populations.

well (e.g. Angers & Bernatchez 1997). Because of their extremely high levels of allelic variability, microsatellites are very useful in population genetic and parentage studies (Bruford & Wayne 1993; Queller *et al.* 1993).

To examine whether populations in the lake are subdivided to such an extent that allopatric speciation can operate at very small geographic scales, we investigated genetic substructuring at six microsatellite loci in four sympatric mbuna species, among four rocky sites separated by sandy bays along 3 km of shore at Nkhata Bay, Malawi (figure 1a).

2. MATERIALS AND METHODS

(a) Sampling

Approximately 50 individuals of each of four *Pseudotropheus* species were sampled at each site in a ca. 300 m² area using SCUBA (table 1). These included two *Pseudotropheus* species from the subgenus *Maylandia* (*P. (M.) zebra* and *P. (M.) callainos*) and two *Pseudotropheus* species from the subgenus *Tropheops* (*P. (T.) 'mauve'* and *P. (T.) 'olive'*) (Ribbink *et al.* 1983; Meyer & Schartl 1984; Trewavas 1984). Specific status of these taxa has been shown by genetic and behavioural data (McKaye *et al.* 1984; van Oppen *et al.* 1998). A 1 cm clipping was taken from the dorsal fin under water. Fin clippings were preserved in NaCl-DMSO buffer (Seutin *et al.* 1991).

(b) DNA extraction and microsatellite analysis

Prior to DNA extraction fin clippings were washed in millipore water and dried on tissue to remove as much of the preservation buffer as possible. DNA was extracted using standard procedures involving proteinase-K digestion, high salt/chloroform extraction and DNA precipitation (Rico *et al.* 1992). DNA was resuspended in 10 mM of Tris-HCl (pH=9.0). Samples were screened for variation at six polymorphic microsatellite loci on the ALF sequencer (Pharmacia) as described in van Oppen *et al.* (1997). Hardly any stutter bands were observed, and in those few instances where stuttering did occur, PCR was repeated until the stutter bands had disappeared (the reason for the occasional stuttering remains unclear). The six loci include four perfect dinucleotide repeats (Pzeb1-3 and UNH002 (Kellogg *et al.* 1996)), one imperfect dinucleotide repeat (Pzeb5) and one compound repeat (Pzeb4). Size of the alleles was calculated from internal standards run in every lane (100 bp+154 bp+259 bp; 100 bp+200 bp+312 bp; 259 bp+353 bp; 100 bp+200 bp; 100 bp+154 bp; and 154 bp+259 bp for the loci Pzeb1, Pzeb2, Pzeb3, Pzeb4, Pzeb5 and UNH002, respectively). Allele sizes were rounded to the nearest integer. Because of this rounding, some alleles will be misclassified as an allele that is one step longer or shorter than the actual size, but this error rate was considered to be acceptably low. Sequencing of 62 alleles of locus Pzeb4 (van Oppen *et al.* 1998) showed that all alleles were sized 1 bp longer than they actually are (which does not affect the size difference between

Table 1. Allele frequencies at the six loci for the four species at the different collection sites from Nkhata Bay

((a) Locus Pzeb1; (b) Locus Pzeb2; (c) Locus Pzeb3; (d) Locus Pzeb4; (e) Locus Pzeb5; (f) Locus UNH002. Abbreviations: Nku, Nkukuti Point; Chir, Chirundu Point; Chin, Chindazowa Point; N, number of alleles sampled.)

(a)

allele length (bp)	<i>P. (M.) zebra</i>				<i>P. (M.) callainos</i>				P. (T.) ‘mauve’				<i>P. (T.) ‘olive’</i>		
	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	
134	—	—	0.011	—	—	—	—	—	—	—	—	—	—	—	
136	0.009	0.019	—	—	—	—	—	0.012	—	—	—	—	—	—	
138	0.009	—	—	—	0.009	—	—	0.085	0.111	0.059	—	—	—	—	
140	—	—	0.023	—	—	—	—	—	0.011	—	0.017	0.028	—	0.008	
142	—	0.01	—	—	—	—	—	0.061	0.078	0.162	—	—	—	—	
144	0.009	—	0.011	0.011	—	—	—	0.037	0.044	0.015	—	—	—	—	
146	—	—	—	—	—	—	—	0.012	0.056	0.029	0.034	—	0.02	0.016	
148	—	0.038	0.091	—	—	—	—	—	0.111	0.059	—	—	—	0.008	
150	0.063	0.058	0.011	—	0.026	0.013	—	0.037	—	0.015	—	0.028	0.02	0.008	
152	0.009	0.029	—	—	—	—	—	0.024	—	—	—	—	—	—	
154	—	—	—	—	—	—	—	0.037	0.022	—	—	0.009	—	—	
156	0.018	—	—	—	—	—	0.009	0.012	0.011	0.015	0.017	0.028	0.01	—	
158	0.009	—	0.045	0.033	0.009	—	0.036	0.037	0.011	—	0.155	0.102	0.15	0.057	
160	0.036	0.029	0.034	0.043	0.052	0.006	0.027	0.024	—	—	0.069	0.111	0.08	0.107	
162	0.045	0.019	0.034	—	—	0.026	0.018	0.012	—	—	0.052	0.074	0.05	0.025	
164	0.098	0.029	0.068	—	0.017	0.006	0.018	0.024	—	—	—	0.009	0.03	0.025	
166	0.071	0.019	0.034	—	—	0.019	0.018	—	—	0.029	0.017	0.046	0.09	0.066	
168	0.027	0.087	0.023	—	0.043	0.032	0.036	—	—	—	0.103	0.009	0.03	—	
170	0.08	0.125	0.023	0.043	0.009	0.019	0.027	—	—	—	—	0.009	—	—	
172	0.036	0.029	0.045	0.011	0.017	0.019	0.027	—	—	—	—	—	0.01	0.008	
174	0.027	0.019	0.034	0.022	0.052	0.032	0.009	—	—	—	—	0.019	0.03	0.033	
176	0.036	0.058	0.045	0.043	—	0.026	—	0.024	0.022	—	0.052	0.046	0.09	0.041	
178	0.054	—	0.034	0.065	0.009	0.019	0.045	—	0.044	0.029	0.017	0.037	0.07	0.008	
180	0.027	0.048	0.034	0.054	0.017	0.038	0.055	0.012	0.089	0.059	0.017	0.009	0.00	0.016	
182	0.071	0.01	0.011	0.022	0.06	0.083	0.055	—	—	0.044	0.052	0.019	0.06	0.074	
184	0.009	0.019	—	0.022	0.026	0.071	0.036	0.037	0.011	0.059	0.034	0.046	0.03	0.049	
186	0.018	0.01	0.034	0.043	0.009	0.026	0.018	0.024	—	—	0.155	0.083	0.13	0.23	
188	0.018	0.019	0.023	0.076	0.043	—	0.009	0.061	—	—	0.172	0.176	0.07	0.139	
190	0.009	0.048	0.034	0.022	0.043	0.032	—	—	0.056	0.029	—	0.037	0.01	0.049	
192	0.054	0.029	0.045	0.011	0.043	0.006	0.027	0.012	0.011	—	—	0.019	0.01	—	
194	—	0.019	—	0.033	0.052	0.013	0.027	0.037	0.078	0.015	—	0.009	—	0.008	
196	0.009	0.019	0.034	0.033	0.017	0.045	0.027	0.024	—	0.029	0.017	—	0.01	0.008	
198	—	—	0.068	0.022	—	0.038	0.036	0.037	0.011	—	0.017	0.019	—	0.016	
200	0.027	0.019	0.045	0.011	0.043	0.019	—	—	0.011	0.029	—	0.009	—	—	
202	0.009	0.019	—	0.022	0.009	0.071	0.009	0.012	0.022	0.015	—	—	—	—	
204	0.009	—	0.023	0.033	0.017	0.013	—	0.037	—	0.029	—	—	—	—	
206	—	—	—	0.011	0.026	0.026	0.045	0.024	—	0.015	—	—	—	—	
208	—	0.01	0.011	0.011	0.034	0.019	0.036	0.024	—	—	—	—	—	—	
210	0.009	0.01	0.011	0.011	0.009	0.026	0.009	0.037	—	0.015	—	—	—	—	
212	0.018	0.019	—	0.065	0.026	0.019	0.018	0.012	0.011	0.044	—	0.009	—	—	
214	0.009	0.048	—	0.043	—	0.019	0.036	—	0.022	0.059	—	—	—	—	
216	0.009	0.01	—	0.033	—	0.019	0.036	0.012	0.033	—	—	—	—	—	
218	0.009	0.029	0.023	—	0.052	0.032	0.055	0.049	—	—	—	—	—	—	
220	0.009	—	—	0.022	0.034	0.019	0.009	0.037	0.011	0.029	—	—	—	—	
222	0.009	0.01	—	—	0.052	0.019	0.009	—	0.011	0.088	—	—	—	—	
224	0.009	0.019	—	0.022	0.017	—	0.027	0.012	0.011	—	—	—	—	—	
226	0.009	0.01	—	0.011	0.017	0.013	0.027	0.024	0.056	—	—	—	—	—	
228	—	—	—	0.022	0.009	—	0.009	—	0.022	—	—	—	—	—	
230	0.009	—	0.011	0.011	0.009	0.013	0.027	—	—	0.015	—	—	—	—	
232	—	0.01	—	0.022	0.009	0.026	0.018	—	0.011	—	—	—	—	—	
234	—	—	0.011	—	0.009	0.006	0.027	—	—	—	—	—	—	—	
236	—	—	0.011	0.011	0.009	—	0.009	—	—	—	—	—	—	—	
238	0.009	—	—	—	—	0.019	0.009	—	—	0.015	—	—	—	—	
240	—	—	—	—	0.017	0.013	0.009	0.012	—	—	—	—	—	—	
242	—	—	—	0.011	0.026	0.026	—	0.024	—	—	—	—	—	—	
244	—	—	—	—	0.009	—	—	—	—	—	—	—	—	—	
246	—	—	—	—	0.017	0.006	—	—	—	—	—	—	—	—	
248	—	—	—	0.011	—	—	0.009	—	—	—	—	—	—	—	

Continued

Table 1 *Continued*

<i>(a) continued</i>															
allele length (bp)	<i>P. (M.) zebra</i>				<i>P. (M.) callainos</i>				<i>P. (T.) 'mauve'</i>				<i>P. (T.) 'olive'</i>		
	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	
250	—	—	—	—	—	0.006	—	—	—	—	—	—	—	—	—
254	—	—	—	—	—	—	—	—	—	—	—	0.009	—	—	—
256	—	—	—	0.011	—	—	—	—	—	—	—	—	—	—	—
<i>N</i>	112	104	88	92	116	156	110	82	90	68	58	108	100	122	
<i>(b)</i>															
allele length (bp)	<i>P. (M.) zebra</i>				<i>P. (M.) callainos</i>				<i>P. (T.) 'mauve'</i>				<i>P. (T.) 'olive'</i>		
	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	
196	—	—	—	0.045	—	—	—	—	—	—	—	—	—	—	—
204	—	0.019	—	—	—	—	—	—	—	—	—	—	—	—	—
206	0.008	—	—	—	—	—	—	—	—	—	—	—	—	—	—
214	—	0.019	0.009	—	—	—	—	—	—	—	—	—	—	—	—
216	—	—	0.009	—	—	—	—	—	—	—	—	—	—	—	—
222	—	—	0.018	—	—	—	—	—	—	—	0.017	—	—	—	—
224	—	0.009	0.018	—	—	—	—	—	—	—	—	—	—	—	—
227	0.056	—	—	—	—	—	—	—	—	—	—	—	—	—	—
229	0.04	0.009	0.036	—	—	—	—	0.048	0.033	0.022	0.017	0.044	0.01	0.009	
231	—	0.009	—	—	—	—	—	0.012	0.011	—	0.05	0.061	0.05	—	—
233	—	—	0.009	—	—	—	—	—	—	0.011	—	—	—	—	—
235	0.032	0.009	—	0.011	—	—	—	—	—	—	—	—	0.01	0.009	
237	—	—	—	0.011	0.018	0.012	0.007	0.012	0.033	—	—	—	—	—	—
239	0.04	0.009	—	—	0.026	—	0.022	0.024	—	—	0.117	0.114	0.17	0.086	
241	0.024	0.009	0.018	0.023	—	—	0.007	0.131	0.065	0.022	—	0.044	—	0.009	
243	0.024	—	0.009	—	0.009	0.019	0.022	0.083	0.043	0.022	0.017	0.018	—	—	—
245	0.024	—	0.009	—	0.009	0.006	0.007	0.024	0.109	0.033	0.017	0.018	—	0.009	
247	0.016	0.056	0.107	0.011	0.018	—	0.052	0.036	0.033	0.033	—	—	—	0.009	
249	0.016	0.046	0.027	0.011	0.018	0.043	0.067	0.012	0.022	0.054	0.017	0.026	0.12	0.095	
251	0.048	—	0.018	0.011	0.053	0.037	0.045	0.083	0.12	0.076	—	0.018	0.02	0.043	
253	—	0.037	0.054	0.045	0.009	0.062	0.075	0.083	0.054	0.076	0.05	0.018	0.02	—	—
255	0.032	0.056	0.054	0.068	0.053	0.043	0.067	0.071	0.043	0.087	0.067	0.009	0.03	0.026	
257	0.048	0.028	0.027	0.057	0.009	0.074	0.03	0.036	0.033	0.087	0.067	0.079	0.12	0.043	
259	0.056	0.065	0.063	0.034	0.079	0.062	0.104	0.036	0.033	0.076	0.117	0.096	0.04	0.052	
261	0.04	0.056	0.018	0.08	0.061	0.105	0.075	0.06	0.076	0.12	0.05	0.026	0.02	0.034	
263	0.111	0.102	0.125	0.045	0.105	0.08	0.112	0.06	0.022	0.054	0.083	0.114	0.15	0.043	
265	0.071	0.065	0.071	0.034	0.14	0.13	0.09	—	0.087	—	—	0.053	0.06	0.095	
267	0.095	0.037	0.045	0.045	0.114	0.111	0.045	0.083	0.043	0.033	—	0.07	0.04	0.103	
269	0.032	0.12	0.063	0.08	0.14	0.062	0.015	0.048	0.054	0.043	0.05	0.035	—	0.078	
271	0.071	0.102	0.036	0.193	0.018	0.068	0.03	0.024	0.033	0.011	—	—	0.04	0.017	
273	0.024	0.037	0.027	0.057	0.061	0.025	0.03	0.012	—	0.043	0.05	0.009	0.04	0.078	
275	0.048	0.009	0.045	0.091	0.044	—	0.015	0.012	0.022	0.054	0.033	0.018	—	0.052	
277	0.024	0.037	0.018	0.034	—	0.025	0.022	0.012	—	0.011	0.017	0.018	0.01	0.052	
279	0.008	0.037	0.045	0.011	0.018	—	0.015	—	0.022	0.033	0.1	0.026	0.01	0.026	
281	0.008	0.009	—	—	—	—	0.037	—	0.011	—	0.017	0.009	0.01	—	—
283	—	0.009	0.009	—	—	0.019	0.007	—	—	—	—	0.018	—	0.017	
285	—	—	—	—	—	0.012	—	—	—	—	0.033	0.035	0.03	0.017	
286	0.008	—	0.009	—	—	0.006	—	—	—	—	—	0.018	—	—	—
288	—	—	—	—	—	—	—	—	—	—	0.017	0.009	—	—	—
292	—	—	0.009	—	—	—	—	—	—	—	—	—	—	—	—
<i>N</i>	126	108	112	88	114	162	134	84	92	92	60	114	100	116	
<i>(c)</i>															
allele length (bp)	<i>P. (M.) zebra</i>				<i>P. (M.) callainos</i>				<i>P. (T.) 'mauve'</i>				<i>P. (T.) 'olive'</i>		
	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	
312	—	—	—	—	—	0.006	—	—	0.065	0.065	0.1	—	—	—	—
314	0.148	0.02	0.173	0.087	0.304	0.222	0.082	0.385	0.446	0.511	0.9	0.754	0.92	0.992	

Table 1 *Continued*

<i>(c) continued</i>															
allele length (bp)	<i>P. (M.) zebra</i>				<i>P. (M.) callainos</i>				<i>P. (T.) 'mauve'</i>				<i>P. (T.) 'olive'</i>		
	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	
316	0.037	0.118	0.058	0.304	0.098	0.057	0.052	0.103	0.065	0.033	—	0.158	0.04	—	
318	0.093	0.078	0.019	0.043	0.143	0.177	0.187	0.051	0.065	0.033	—	0.044	0.04	0.008	
320	0.481	0.373	0.462	0.315	0.313	0.348	0.552	0.321	0.348	0.326	—	0.026	—	—	
322	0.139	0.304	0.173	—	0.08	0.013	0.052	0.064	0.011	0.033	—	0.018	—	—	
324	0.019	0.059	0.01	0.054	0.018	0.044	—	0.026	—	—	—	—	—	—	
326	0.037	0.029	0.029	—	0.009	0.006	—	0.013	—	—	—	—	—	—	
328	0.009	0.01	0.01	0.087	0.009	0.051	0.06	0.026	—	—	—	—	—	—	
330	0.019	—	0.038	—	0.018	0.032	0.015	—	—	—	—	—	—	—	
332	—	—	0.019	0.022	0.009	0.013	—	—	—	—	—	—	—	—	
334	0.019	—	—	0.043	—	0.019	—	0.013	—	—	—	—	—	—	
336	—	—	—	0.033	—	—	—	—	—	—	—	—	—	—	
338	—	0.01	—	0.011	—	0.013	—	—	—	—	—	—	—	—	
362	—	—	0.01	—	—	—	—	—	—	—	—	—	—	—	
<i>N</i>	108	102	104	92	112	158	134	78	92	92	60	114	100	122	
<i>(d)</i>															
allele length (bp)	<i>P. (M.) zebra</i>				<i>P. (M.) callainos</i>				<i>P. (T.) 'mauve'</i>				<i>P. (T.) 'olive'</i>		
	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	
117	—	—	0.009	—	—	—	—	—	—	—	—	—	—	—	
121	—	—	0.017	—	—	—	—	—	—	—	—	—	—	0.017	
123	0.263	0.316	0.345	0.136	0.116	0.075	0.169	0.756	0.685	0.822	0.283	0.136	0.163	0.28	
125	—	0.01	0.069	—	—	—	—	0.024	0.13	0.056	—	—	—	—	
127	0.339	0.306	0.353	—	—	—	—	—	—	—	—	—	0.051	—	
129	0.017	—	0.017	—	—	0.087	0.016	—	—	0.011	—	—	0.01	—	
131	0.093	0.133	0.06	0.432	0.438	0.538	0.452	0.22	0.185	0.089	0.05	0.009	0.143	0.093	
133	0.136	0.102	0.086	0.42	0.446	0.3	0.339	—	—	0.011	0.65	0.855	0.633	0.61	
135	—	—	—	—	—	—	0.008	—	—	0.011	—	—	—	—	
137	0.093	0.082	0.009	0.011	—	—	0.016	—	—	—	—	—	—	—	
139	0.017	0.02	0.017	—	—	—	—	—	—	—	—	—	—	—	
141	0.008	—	0.009	—	—	—	—	—	—	—	0.017	—	—	—	
143	0.017	0.01	0.009	—	—	—	—	—	—	—	—	—	—	—	
145	0.017	0.02	—	—	—	—	—	—	—	—	—	—	—	—	
<i>N</i>	118	98	116	88	112	160	124	82	92	90	60	110	98	118	
<i>(e)</i>															
allele length (bp)	<i>P. (M.) zebra</i>				<i>P. (M.) callainos</i>				<i>P. (T.) 'mauve'</i>				<i>P. (T.) 'olive'</i>		
	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	
121	0.026	—	0.009	—	—	—	—	0.012	—	0.011	—	—	—	—	
123	0.052	0.065	0.036	0.433	0.552	0.392	0.455	0.023	0.033	0.011	—	—	—	—	
125	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
127	0.026	—	—	—	—	—	—	—	—	—	—	—	—	0.017	
129	0.741	0.833	0.83	0.567	0.448	0.608	0.538	0.93	0.957	0.967	0.931	0.956	0.94	0.915	
131	0.009	—	0.054	—	—	—	0.008	0.035	0.011	0.011	0.017	0.009	0.03	0.008	
135	—	—	—	—	—	—	—	—	—	—	0.017	0.018	0.01	0.017	
137	0.147	0.102	0.071	—	—	—	—	—	—	—	0.034	0.018	0.02	0.042	
<i>N</i>	116	108	112	90	116	148	132	86	92	90	58	114	100	118	
<i>(f)</i>															
allele length (bp)	<i>P. (M.) zebra</i>				<i>P. (M.) callainos</i>				<i>P. (T.) 'mauve'</i>				<i>P. (T.) 'olive'</i>		
	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	
168	—	—	—	—	—	—	—	—	0.022	0.011	—	—	—	—	
174	0.157	0.057	0.009	—	0.018	—	0.015	—	0.022	—	—	—	—	—	

Continued

Table 1 *Continued*

(f) *continued*

allele length (bp)	<i>P. (M.) zebra</i>				<i>P. (M.) callainos</i>				P. (T.) ‘mauve’				<i>P. (T.)</i> ‘olive’		
	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	Nku	Chir	Chin	Mwa	Nku	Chir	Chin	
179	0.019	—	—	—	—	—	—	—	—	—	—	—	—	—	
181	—	—	—	0.022	—	—	—	—	—	—	—	—	—	—	
183	0.009	0.019	—	—	—	—	—	—	—	—	—	—	—	—	
185	—	—	—	—	—	—	—	0.035	0.033	0.1	0.033	—	0.03	—	
187	—	—	0.009	—	—	—	—	0.023	—	—	0.033	0.018	—	—	
189	0.009	—	—	—	0.009	0.014	0.008	—	—	—	—	0.035	—	0.017	
191	—	—	—	0.054	0.035	0.02	0.008	—	—	—	0.017	0.018	—	0.025	
193	0.009	0.019	0.036	0.043	0.018	0.027	0.031	—	0.011	—	—	0.053	0.01	0.017	
195	0.009	0.028	0.018	0.109	0.018	0.02	—	0.012	0.022	0.022	—	0.009	—	—	
197	0.046	—	—	0.065	0.018	—	0.038	0.023	0.011	—	—	—	—	—	
199	0.009	0.028	—	0.043	0.035	0.068	0.023	0.14	0.25	0.256	—	0.009	—	—	
201	0.009	0.009	0.073	0.022	0.026	0.007	0.054	0.035	0.054	0.067	—	0.026	0.03	0.025	
203	0.028	0.057	0.1	0.033	0.061	0.047	0.077	0.012	0.022	0.044	—	—	—	—	
205	0.009	0.009	0.009	0.076	0.053	0.081	0.038	0.105	0.13	0.067	—	—	—	—	
207	0.102	0.038	0.109	—	0.105	0.02	0.031	—	—	—	0.05	0.07	0.05	0.042	
209	0.019	0.085	0.064	0.022	0.061	0.007	0.077	0.058	0.065	0.056	0.017	—	0.03	0.033	
211	0.028	0.094	0.036	0.065	0.053	0.068	0.015	—	0.011	0.011	0.083	0.009	—	0.075	
213	0.009	0.028	0.009	0.152	—	0.02	0.031	0.023	—	0.022	0.017	0.018	0.05	0.033	
215	0.028	0.009	0.036	0.022	—	0.054	0.092	0.012	—	—	—	0.07	—	0.033	
217	—	0.066	0.045	0.033	0.07	0.149	0.077	—	—	0.011	0.183	0.342	0.38	0.308	
219	0.028	—	0.009	0.022	0.061	0.074	0.031	—	—	—	0.2	0.149	0.11	0.1	
221	0.009	—	0.018	—	0.07	0.061	0.038	0.023	—	—	0.2	0.035	0.11	0.092	
223	—	0.028	0.009	—	0.07	0.034	0.077	0.163	0.033	0.033	—	0.035	0.03	0.067	
225	0.019	0.009	0.073	0.033	0.053	0.054	0.069	0.221	0.163	0.233	0.033	0.009	0.01	0.042	
227	0.074	0.009	0.036	0.109	0.026	0.061	0.062	0.116	0.12	0.033	—	—	0.03	0.025	
229	0.028	0.085	0.027	0.033	0.061	0.027	0.023	—	0.022	0.022	—	0.018	0.02	0.017	
231	0.046	0.094	0.109	0.022	0.026	0.02	0.023	—	—	0.011	—	0.009	—	—	
233	0.065	0.038	0.055	—	0.018	0.027	0.015	—	—	—	—	—	—	—	
235	0.074	0.028	0.009	—	—	0.007	0.008	—	—	—	0.1	0.018	0.05	0.017	
237	0.028	0.019	0.009	—	0.009	0.014	—	—	—	—	0.033	0.053	0.06	0.033	
239	0.009	0.028	0.018	—	—	—	0.015	—	0.011	—	—	—	—	—	
241	0.028	0.009	0.009	—	0.018	0.014	0.023	—	—	—	—	—	—	—	
243	0.037	0.057	0.036	0.022	—	0.007	—	—	—	—	—	—	—	—	
245	0.009	—	0.027	—	0.009	—	—	—	—	—	—	—	—	—	
247	0.037	0.009	—	—	—	—	—	—	—	—	—	—	—	—	
249	0.009	—	—	—	—	—	—	—	—	—	—	—	—	—	
255	—	0.019	—	—	—	—	—	—	—	—	—	—	—	—	
257	—	0.019	—	—	—	—	—	—	—	—	—	—	—	—	
<i>N</i>	108	106	110	92	114	148	130	86	92	90	60	114	100	120	

alleles), but that only two alleles were wrongly sized, giving an error rate of 3%. It is likely that the other five loci have a similar error rate, since sizing was performed in the same manner. Of locus Pzebl, the original allele (226 bp) that was sequenced from the genomic library had a (GT)₅₉ repeat (van Oppen *et al.* 1997). Another allele (142 bp) (M. J. H. van Oppen, unpublished data) has only 17 GT repeats, suggesting that sizing has generally been correct.

(c) Data analysis

Allele frequencies were calculated and genetic substructuring was estimated by calculating fixation indices assuming either the infinite allele model (IAM; θ_{st} , Weir & Cockerham (1984)) using GENEPOP 1.2 (Raymond & Rousset 1995) and FSTAT 1.2 (Goudet 1995) or the single-step, stepwise mutation model (SMM) using WINAMOVA

1.5 (ϕ_{st} , Michalakis & Excoffier (1996)). To test whether fixation indices departed significantly from zero, individuals (genotypes) were permuted 1000 times, and fixation indices were calculated for each of the 1000 permuted data sets. The *p* value calculated gives the fraction of those 1000 permutations in which the fixation index is lower than the initially observed value. The number of migrants per generation (*N_m*) was calculated using the private allele method (Slatkin 1985; Allen *et al.* 1995).

The approximate number of adult territorial males (a very rough field estimate of effective population size), *N_t*, was estimated on one of the four habitat patches (Nkukuti Point), calculated from densities at Nkukuti Point published Ribbink *et al.* (1983) and depth contours in the 1954 Malawi Hydrographic Survey 'Nkata Bay' chart. Densities in Ribbink *et al.* (1983) were obtained by counting all territorial males of a particular species present along a 25 m line transect (2 m wide) at different depths using SCUBA.

Table 2. Average θ_{st} and ϕ_{st} over all loci and all subpopulations, numbers of migrants per generation (N_m), and estimates of numbers of territorial males per population (N_t) for the four *Pseudotropheus* species

	<i>P. (M.) zebra</i>	<i>P. (M.) callainos</i>	<i>P. (T.) 'mauve'</i>	<i>P. (T.) 'olive'</i>
θ_{st}	0.008**	0.015**	0.007*	0.016**
ϕ_{st}	0.000a	0.002**	0.012**	0.020**
N_m	7.5	8.3	6.3	7.0
N_t	15 000	18 000	6000	2500

^a Not significant, * $0.001 < p \leq 0.01$, ** $p \leq 0.001$.

(d) Modelling

Following Jin & Chakraborty's (1995) formulations, we have modelled the expected levels of genetic differentiation (using G_{st} as a measure) for ten subpopulations of equal effective population size (N_e) which have not exchanged any genes for t generations. The subpopulations are assumed to be at Hardy–Weinberg equilibrium with $H_s = 0.9$ corresponding to the average expected heterozygosity at the more polymorphic loci of our sample populations (see §3). For the IAM, G_{st} as a function of the number of subpopulations (s), the within subpopulation heterozygosity (H_s) and the divergence time (t) among the subpopulations (in generations) is given by:

$$G_{st} = \frac{(1 - s^{-1})(1 - H_s)[1 - e^{-H_s T/(1-H_s)}]}{H_s + (1 - s^{-1})(1 - H_s)[1 - e^{-H_s T/(1-H_s)}]}$$

where $T = 1/2 N_e$ and N_e is the effective subpopulation size (Chakraborty & Jin 1992; Jin & Chakraborty 1995). The analogous function for the single-step (SMM) is:

$$G_{st} = \frac{(s-1)(1-H_s-\bar{f}_{xy})}{sH_s + (s-1)(1-H_s-\bar{f}_{xy})}$$

where $\bar{f}_{xy}$ the probability that two genes drawn randomly, one from each of two subpopulations x and y , are identical is given by:

$$\bar{f}_{xy} = e^{-MT} \sum_{i=-\infty}^{\infty} \left[\left[\frac{1 + M - (1 - H_s)^{-1}}{M} \right]^i (1 - H_s) I(i, MT) \right],$$

$$\text{and } M = \frac{[(1 - H_s)^{-2} - 1]}{2},$$

and $I(i, MT)$ is the i th order ($0 \leq i \leq \infty$) modified Bessel function of the first kind.

3. RESULTS

For three of the four species studied, overall genetic differentiation among the allopatric populations based on all six microsatellite loci was highly significant (table 2), indicating that some degree of isolation between populations is present. For the fourth species (*P. (M.) zebra*), significance depended on which evolutionary model was used. Allele frequency distributions at several of the loci differed markedly between sites (table 1), although the frequency of microsatellite alleles unique for a single site was generally low (see figure 1b for an example). Fixation indices were low,

which is to be expected at highly polymorphic loci exhibiting many alleles and high heterozygosities (Jin & Chakraborty 1995). Figure 2 shows the expected values of the coefficient of gene diversity, G_{st} , for various effective population sizes and divergence times assuming no gene flow under the IAM (figure 2a) and the single-step SSM (figure 2b). Expected G_{st} values following the IAM are twice as high as those from the single-step SMM.

Expected heterozygosity averaged over the four species was 0.95, 0.94, 0.74, 0.56, 0.26 and 0.91 for locus Pzeb1, Pzeb2, Pzeb3, Pzeb4, Pzeb5 and UNH002, respectively. Observed heterozygosities are significantly lower for Pzeb1, Pzeb2, Pzeb3 and UNH002, suggesting the presence of non-amplifying null alleles. Pedigree analysis has shown that null alleles are indeed present in Pzeb1, Pzeb2, Pzeb3 and UNH002, but not in Pzeb4 and Pzeb5 (van Oppen *et al.* 1998a). This is not expected to influence estimates of genetic diversification appreciably, since significant θ_{st} values were found in three of the four species ($\theta_{st} = 0.011$, $p = 0.019$; $\theta_{st} = 0.016$, $p = 0.026$; and $\theta_{st} = 0.031$, $p = 0.001$ for *P. (M.) callainos*, *P. (T.) 'mauve'* and *P. (T.) 'olive'*, respectively) using only the two loci with no heterozygote deficiency.

Effective numbers of migrants (estimated from private alleles) between populations varied between 6.3 and 8.3 individuals per generation for the different species and sites (table 2). Numbers of adult territorial males were estimated to range between 2500 and 18 000 for the different species (table 2).

4. DISCUSSION

Highly significant levels of genetic differentiation were found in the four mbuna species investigated, suggesting that gene flow is limited between the Nkhata Bay populations. On the face of it, the levels of genetic differentiation attained by the populations seem low (table 2), especially in the light of their high statistical significance. Similar or even lower, but statistically highly significant, estimates have been reported for other taxa (e.g. Allen *et al.* 1995; García de León *et al.* 1997) and we have also found similarly low θ_{st} values between some sympatric *Pseudotropheus* species which mate assortatively in the field (van Oppen *et al.* 1998). Jin & Chakraborty (1995) have shown that scores of genetic differentiation based on hypervariable loci with large numbers of alleles are expected to be much smaller compared to those based on the more traditional blood group and protein loci. Moreover, it is unlikely that the Nkhata Bay populations have been diverging for more than 200 years. Today's habitat patches probably did not exist in their present form 200 years ago, when lake water levels were 120 m lower than they are now (Owen *et al.* 1990). Ribbink *et al.* (1983) report that about 70% of the rocky shores do not extend beyond 40 m in depth. It is unlikely that all four Nkhata Bay patches investigated here extend to depths of 120 m, and even if they do, it is then unlikely that all four of them remain separated over the whole depth trajectory. Thus, assuming that these rocky outcrops were colonized from a homogeneous source,

Figure 2. Expected values of the coefficient of gene diversity, G_{st} , for various effective population sizes (N_e , each square on the z -axis in the graph represents a step of *ca.* 771 individuals) and divergence times (in number of generations, each square on the x -axis represents a step of 16.7 generations) assuming no gene flow under the infinite allele model (a) and the single-step, stepwise mutation model (b).

the populations could have only diverged for 200 years or approximately 67 generations, taking the mean age at reproduction of females in these age-structured populations (Charlesworth 1980) to be three years. We have assumed that *Pseudotropheus* species, like most other Malawian haplochromines studied in the field (Tweddle & Turner 1977), mature at three years, although unpublished observations of laboratory stocks indicate that they mature at one year at a smaller size than has been observed in the field.

If it is assumed that the level of genetic differentiation between these sites is currently maintained at equilibrium by migration, mutation, and genetic drift, then the numbers of migrants between populations varies between 6.3 and 8.3 individuals per generation for the different species and sites (table 2). Migration rates higher than 1 do not allow fixation of alternative alleles in the different populations (Wright 1969). It is quite likely, however, that these populations have been isolated for an insufficient time (*ca.* 67 generations) to reach equilibrium and, as a result, the migration rates are overestimated. Figure 2 shows that even when N_e is as low as 500, some 300 generations are required before G_{st} reaches asymptotic values, indicating equilibrium conditions. Despite this, the estimates of number of migrants (table 2) represent a surprisingly low level of migration between adjacent populations of 2500–18 000 adult territorial males.

Figure 2 shows that the observed level of genetic differentiation between these populations is approximately equal to that expected for populations with an N_e of 2300–3000 that have been completely isolated from each other for 67 generations. This is at the lower end of the estimate of numbers of territorial males shown in table 2, which can be taken as a very rough estimate of N_e . In making these inferences, it is important to bear in mind that among the assumptions of the model are origin of the populations from a homogeneous pool, absence of selection, and comparable population sizes. Since actual population sizes are likely to be much higher than the lower end estimates

suggested by the model, some sort of selection, possibly sexual, could be contributing to the observed differentiation (Payne & Krakauer 1997). All these factors support the possibility of recent isolation with little or no current gene flow.

To find allopatric subdivision at such a small geographic scale is extremely unusual for fishes inhabiting a single lake or sea with no obvious barriers to dispersal. Although many freshwater fishes show strong genetic partitioning due to the land barriers separating drainages or colonization of the rivers from different refugia (Avice 1994, and references therein), most marine fishes exhibit low levels of genetic divergence (e.g. Shaklee 1984; Lacson 1992; Shulman & Bermingham 1995; Rico *et al.* 1997), even at vast geographical scales (greater than 1000 km). Some examples of relatively small-scale biogeographic subdivision in marine fishes are known (Bell *et al.* 1982; Avice 1994, and references therein), but the scale of substructuring is still far greater than in the cichlid example we present here. No simple correlation seems to exist between length of the pelagic larval phase or egg type and the extent of genetic substructuring (Lacson 1992; Avice 1994; Shulman & Bermingham 1995). Nevertheless, it seems likely that maternal mouthbrooding in conjunction with the nature of the shores of Lake Malawi (rocky outcropping alternating with sandy bays) is responsible for the extraordinary degree of fine-scale genetic substructuring found in this study.

The unusually fine-scale geographic subdivision of these cichlid populations may partly explain their explosive speciation, allowing divergence amongst these allopatric populations by genetic drift, adaptation to changing habitats, or sexual selection. Circumstantial evidence and modelling (Turner & Burrows 1995) suggests that sexual selection may be involved: the most apparent differences between closely related species and allopatric populations are in male breeding colours (Ribbink *et al.* 1983), and the highly polygynous breeding system with no male parental care provides ample opportunity for female mate choice. Female

choice has indeed been demonstrated (Hert 1989, 1991). Future work should attempt to elucidate the causes of speciation.

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